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Signaling requirements and role of salicylic acid in *HRT*- and *rrt*-mediated resistance to turnip crinkle virus in Arabidopsis

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Summary

Inoculation of turnip crinkle virus (TCV) on the resistant *Arabidopsis* ecotype Di-17 elicits a hypersensitive response (HR), which is accompanied by increased expression of pathogenesis-related (*PR*) genes. Previous genetic analyses revealed that the HR to TCV is conferred by *HRT*, which encodes a coiled-coil (CC), nucleotide-binding site (NBS) and leucine-rich repeat (LRR) class resistance (R) protein. In contrast to the HR, resistance to TCV requires both *HRT* and a recessive allele at a second locus designated *rrt*. Here, we demonstrate that unlike most CC-NBS-LRR *R* genes, *HRT/rrt*-mediated resistance is dependent on *EDS1* and independent of *NDR1*. Resistance is also independent of *RAR1* and *SGT1*. *HRT/rrt*-mediated resistance is compromised in plants with reduced salicylic acid (SA) content as a consequence of mutations *eds5*, *pad4*, or *sid2.* By contrast, HR is not affected by mutations in *eds1*, *eds5*, *pad4*, *sid2*, *ndr1*, *rar1*, or *sgt1b.* Resistance to TCV is restored in both SA-deficient Di-17 plants expressing the *nahG* transgene and mutants containing the *eds1*, *eds5*, or *sid2* mutations by exogenous application of SA or the SA analog benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH). In contrast, SA/BTH treatment failed to enhance resistance in *HRT pad4*, Col-0, or *hrt* homozygous progeny of a cross between Di-17 and Col-0. Thus, HRT and PAD4 are required for SA-induced resistance. Exogenously supplied SA or high endogenous levels of SA, due to the *ssi2* mutation, overcame the suppressive effects of *RRT* and enhanced resistance to TCV, provided the *HRT* allele was present. High levels of SA upregulate *HRT* expression via a *PAD4*-dependent pathway. As Col-0 transgenic lines expressing high levels of *HRT* were resistant to TCV, but lines expressing moderate to low levels of *HRT* were not, we conclude that SA enhances resistance in the *RRT* background by upregulating *HRT* expression. These data suggest that the HRT-TCV interaction is unable to generate sufficient amounts of SA required for a stable resistance phenotype, and the presence of *rrt* possibly corrects this deficiency.

Keywords: turnip crinkle virus, salicylic acid, defense, *Arabidopsis*, signaling.

Introduction

Plants have evolved various defense mechanisms to resist pathogen infection. Recognition of an invading pathogen by the host plant often involves interaction between a plant resistance (*R*) gene and a pathogen avirulence (*avr*) gene (Flor, 1971). Upon recognition, the host plant initiates one or more signal transduction pathways that activate various plant defenses, thereby averting pathogen colonization. In many cases, resistance is associated with increased expression of defense genes, including the pathogenesis-related (*PR*) genes and the accumulation of salicylic acid (SA) in the inoculated leaf; localized host cell death at the site of pathogen entry, a phenomenon known as the hypersensitive response (HR), also occurs. Subsequent to the HR, the uninoculated tissues of the plant usually develop a long-lasting, enhanced resistance to further attack by the same or unrelated pathogens. This phenomenon, known as systemic acquired resistance, is accompanied by a systemic increase in the levels of SA and *PR* gene expression (Dempsey *et al.*, 1999; Durrant and Dong, 2004).

Many *R* genes that provide protection against various pathogens have been cloned, and these can be broadly classified into five categories (Dangl and Jones, 2001; Ellis *et al.*, 2000). The largest class encodes proteins containing nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains. The NBS–LRR genes can be subdivided further based on whether they have a coiled-coil (CC) domain or a toll-interleukin-1 receptor (TIR)-like region at their N-terminus. The CC and TIR domains are believed to relay the pathogen-perceived signals; the resistance signaling pathways downstream of these proteins are generally dependent on the products of the *NDR1* or *EDS1* genes, respectively (Aarts *et al.*, 1998; Dangl and Jones, 2001). Defense signaling activated by various *R*–*avr* interactions is thought to converge at a point further downstream, resulting in the activation of a common set of defense genes (Dong, 2001).

Salicylic acid plays a critical signaling role in the activation of disease resistance in plants (Dempsey *et al.*, 1999; Durrant and Dong, 2004). When SA accumulation is suppressed in tobacco and *Arabidopsis* by expression of the *nahG* transgene, which encodes the SA-degrading enzyme SA hydroxylase, susceptibility to both compatible and incompatible pathogens is enhanced and *PR* gene expression is suppressed (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). Similarly, *Arabidopsis* mutants that are impaired in SA perception, such as *npr1* (Cao *et al.*, 1994; Ryals *et al.*, 1997; Shah *et al.*, 1997), or pathogen-induced SA accumulation, such as *eds1* (Falk *et al.*, 1999), *eds5* (Nawrath and Metraux, 1999; Nawrath *et al.*, 2002), *sid2* (Wildermuth *et al.*, 2001), and *pad4* (Jirage *et al.*, 1999), exhibit enhanced susceptibility to pathogen infection and impaired *PR* gene expression. As pathogen-induced expression of *EDS5* is impaired in *eds1* and *pad4* mutants, EDS5 appears to function downstream of EDS1 and PAD4 in the defense signaling pathway (Nawrath *et al.*, 2002).

The mechanism through which EDS1 (a putative lipase; Falk *et al.*, 1999), EDS5 (a member of the MATE transporter family; Nawrath *et al.*, 2002), and PAD4 (a putative lipase; Jirage *et al.*, 1999) regulate pathogen-induced SA accumulation is unclear. However, the discovery that *SID2* encodes isochorismate synthase argues that plants utilize the chorismate pathway for SA biosynthesis (Wildermuth *et al.*, 2001). In addition, evidence from several studies suggests that SA is also synthesized from phenylalanine (Mauch-Mani and Slusarenko, 1996; Pallas *et al.*, 1996; Ribnicky *et al.*, 1998).

We previously isolated a line, designated Di-17, from the Dijon (Di-0) ecotype that consistently develops an HR, expresses *PR* genes and accumulates SA in response to inoculation with turnip crinkle virus (TCV). The majority of these plants (85–100%) also exhibit TCV resistance, based on their ability to restrict the virus to inoculated leaves (Dempsey *et al.*, 1993, 1997; Kachroo *et al.*, 2000; Simon *et al.*, 1992). TCV-induced cell death and *PR* gene expression were conferred by *HRT*, which encodes a putative R protein with CC–NBS and LRR-like domains (At5g43470; Cooley *et al.*, 2000). *HRT* is also required for TCV resistance; however, it is insufficient in the absence of a recessive allele at a second locus designated *rrt* (Kachroo *et al.*, 2000). The HR and resistance phenotypes are dependent on SA, but do not require *NPR1*-, ethylene-, or jasmonic acid-mediated defense signaling pathways (Kachroo *et al.*, 2000). In comparison, resistance conferred by *HRT* allelic genes *RPP8* and *RCY1* against *Peronospora parasitica* biotype Emco5 and cucumber mosaic virus, respectively, are SA-independent and partially SA-dependent pathways (McDowell *et al.*, 1998; Takahashi *et al.*, 2002). These observations suggest that a high level of structural similarity between *HRT*, *RPP8*, and *RCY1* genes does not necessitate an overlap in the requirement for downstream signaling components.

In the present work, we demonstrate that HRT activates resistance via a pathway that is dependent on *EDS1*, *EDS5*, *PAD4*, and *SID2*, but independent of *NDR1*, *RAR1*, and *SGT1*. As TCV-induced *PR* gene expression and HR formation are not affected by mutations in these genes, *HRT*-mediated resistance appears to be activated via a distinct pathway from *HRT*-induced *PR* expression and cell death. Analysis of *HRT eds1*, *HRT eds5*, *HRT pad4*, and *HRT sid2* plants revealed that they accumulate reduced levels of SA following TCV infection. Thus, a critical level of SA appears to be required to signal TCV resistance. The observation that SA/benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) treatment only enhanced resistance in plants containing *HRT*, however, argues that SA does not function downstream of HRT, but rather, in conjunction with it. A model for the HRT signaling pathway and its interaction with the RRT suppressor is presented.

Results

HRT*-mediated resistance is independent of* NDR1*,* RAR1 *and* SGT1*, but dependent on* EDS1

To investigate the signaling components involved in the *HRT* signaling pathway, we crossed Dijon (Di-17) with Columbia (Col-0) plants containing mutations in the *R* gene signal transducers *ndr1-1* (Col-0 background) or *eds1-1* [Wassilewskija (Ws) background]. The effect of the *rar1* and *sgt1b* mutations (Landsberg background) was also assessed as these genes are required to signal responses mediated by a diverse range of *R* genes and therefore may serve as points of convergence for various R protein-triggered pathways (Austin *et al.*, 2002; Azevedo *et al.*, 2002; Liu *et al.*, 2002; Muskett and Parker, 2003; Muskett *et al.*, 2002; Tör *et al.*, 2002). Analysis of *HRT*-containing F_2 plants homozygous for the mutant loci failed to detect any difference in HR development or *PR-1* expression following TCV inoculation (Figure 1a,b, Table 1). It should be noted that due to tight linkage between *RAR1* and *HRT*, only five *HRT rar1* plants were obtained.

Whether disease resistance was affected in the various F_2 progeny was then assessed. As resistance segregates in a recessive manner (Kachroo *et al.*, 2000), 25% of the F_2 plants

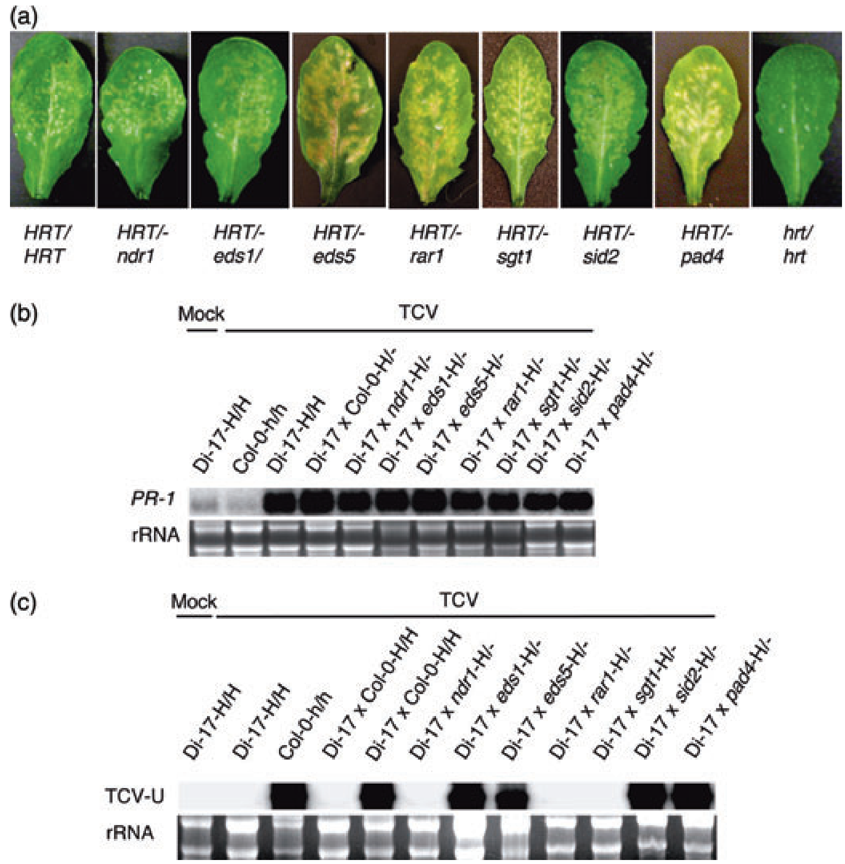

Figure 1. Effect of *ndr1*, *eds1*, *eds5*, *rar1*, *sgt1b*, *sid2*, and *pad4* mutations on the *HRT*-mediated hypersensitive response (HR), *PR-1* expression and resistance to turnip crinkle virus (TCV).
(a) Cell death in TCV-inoculated leaves of F_2 progeny from various crosses at 3 or 4 DPI. F_2 progeny were homozygous for the mutant locus and had at least one copy of the *HRT* gene. The small specks seen on the inoculated leaves of the *hrt/hrt* plants are dried inoculation buffer.
(b) *PR-1* gene expression in Di-17, Col-0, and F_2 progeny derived from crosses between Di-17 and various mutants, inoculated with TCV or buffer alone. The F_2 progeny were homozygous for the mutant allele and had at least one copy of the *HRT* gene (H/-). Ethidium bromide staining of rRNA was used as a loading control.
(c) Resistance and systemic spread of TCV in various genotypes shown in (b) and the F_2 progeny of Di-17 × Col-0. Both Di-17 × Col-0 F_2 progeny were homozygous for *HRT* but only one of these genotypes was resistant to TCV and did not allow systemic spread of the virus. RNA was extracted from the uninoculated tissues at 20 DPI and analyzed for the presence of viral transcripts. Ethidium bromide staining of rRNA was used as a loading control.

containing at least one dose of *HRT* should be resistant to TCV if the mutant locus is not required. Consistent with this hypothesis, a ratio of approximately 3 susceptible:1 resistant plant was observed in the *HRT ndr1*, *HRT sgt1b* and *HRT rar1* F_2 progeny, as well as in the F_2 progeny of a Di-17 × Col-0 cross (Table 1, Figure 1c). In comparison, all of the *HRT eds1* plants were susceptible to TCV, evidenced by systemic spread of the virus (Table 1, Figure 1c). Taken together, these results suggest that *HRT*-mediated cell death and *PR-1* gene expression are independent of *NDR1*, *EDS1*, *RAR1*, and *SGT1*, whereas resistance to TCV is independent of *NDR1*, *RAR1*, and *SGT1*, but dependent on *EDS1*.

The HRT*-mediated cell death, but not resistance, is independent of* PAD4*,* EDS5*, and* SID2

Earlier results demonstrated that *HRT*-mediated HR and resistance are abolished in the NahG background (Kachroo *et al*., 2000), suggesting that SA is required for both of these phenotypes. To further investigate the role of SA in the *HRT* signaling pathway, Di-17 plants were crossed with *pad4-1*, *eds5-1*, or *sid2-1* mutants (Col-0 background), which display impaired SA accumulation following pathogen infection (Jirage *et al*., 1999; Nawrath and Metraux, 1999; Wildermuth *et al*., 2001). Following identification of F_2 plants homozygous for the mutant alleles and containing at least one copy of *HRT*, cell death, *PR-1* expression, and TCV resistance were assessed. Neither cell death nor *PR-1* expression was affected in *HRT pad4*, *HRT eds5*, or *HRT sid2* F_2 progeny (Figure 1a,b). By contrast, all three mutations abolished TCV resistance (Table 1, Figure 1c). Based on these results, *HRT*-mediated TCV resistance, but not cell death or *PR-1* gene expression is dependent on *PAD4*, *EDS5*, and *SID2*.

RRT *is not linked to* EDS1*,* PAD4*,* EDS5*, and* SID2 *loci*

Previous analyses demonstrated that TCV resistance is dependent not only on *HRT*, but also on a recessive locus, *rrt* (Kachroo *et al*., 2000). As the map position of *RRT* is not yet known, it is conceivable that a linkage between *RRT* and any of the mutant loci would cause skewed segregation, which is independent of any affect exerted by the mutant locus. If a linkage leads to increased susceptibility among *HRT* plants that are homozygous for the mutant loci (and as a result *RRT/RRT*) it should also increase the number of resistant plants among F_2 population that contain *HRT* and have wild-type genotype at the mutant locus (Di-17 genotype and thus *rrt/rrt*). An expected ratio of approximately 3 susceptible to 1 resistance plant was observed in F_2 plants that were *HRT*/- and *EDS1/EDS1*, *PAD4/PAD4*, *EDS5/EDS5* or *SID2/SID2*

Table 1 Epistatic analysis of F_2 population derived from crosses between Di-17 and various wild-type or mutant lines

Cross	Total number of plants analyzed	Genotype[a]	Number of plants obtained	HR[b]	R[c]	S[d]	χ^2	P[e]
Di-17 × Col-0	124	*HRT/-*	30	+	7	23	0.046	0.83
Di-17 × Ws	115	*HRT/-*	26	+	5	21	0.45	0.50
Di-17 × *eds1*	349	*HRT/-eds1/eds1*	60	+	0	60	20.0	<0.001[h]
Di-17 × *eds1*	125	*HRT/-eds1/eds1*	21	+	0	21	7.0	0.0082[h]
		HRT/-EDS1/EDS1	22	+	3	19	1.5	0.22
Di-17 × *ndr1*	150	*HRT/-ndr1/ndr1*	33	+	7	26	0.28	0.59
Di-17 × *eds5*	164	*HRT/-eds5/eds5*	39	+	0	39	12.99	<0.001[h]
Di-17 × *eds5*	130	*HRT/-eds5/eds5*	26	+	0	26	8.67	0.0032[h]
		HRT/-EDS5/EDS5	24	+	5	19	0.22	0.63
Di-17 × *sgt1b*	134	*HRT/-sgt1b/sgt1b*	30	+	6	24	0.4	0.52
Di-17 × *rar1*	276	*HRT/-rar1/rar1*	5	+	1[f]	4	ND[g]	ND
Di-17 × *sid2*	217	*HRT/-sid2/sid2*	30	+	0	30	10.0	<0.001[h]
Di-17 × *sid2*	218	*HRT/-sid2/sid2*	26	+	0	26	8.66	0.0033[h]
		HRT/-SID2/SID2	41	+	9	32	0.20	0.65
Di-17 × *pad4*	224	*HRT/-pad4/pad4*	43	+	0	43	13.83	0.0002[h]
Di-17 × *pad4*	122	*HRT/-pad4/pad4*	18	+	0	18	6.0	0.014[h]
		HRT/-PAD4/PAD4	22	+	4	18	0.53	0.46

[a]The genotype at *HRT* and various mutant loci was determined by CAPS analysis.
[b]HR, hypersensitive response.
[c]Resistant.
[d]Susceptible.
[e]One degree of freedom.
[f]Resistance was confirmed by inoculating 36 F_3 plants; all were resistant.
[g]Not determined.
[h]Statistically significant.

(Table 1). This indicates that *RRT* is not linked to any of these loci and a susceptible response seen in the *HRT eds1*, *HRT pad4*, *HRT eds5*, and *HRT sid2* plants is due to impaired signaling.

Mutations in eds1*,* pad4*,* eds5*, and* sid2 *lower SA levels in TCV-inoculated* HRT *plants*

As resistance to TCV was suppressed by mutations in *pad4-1*, *eds5-1* and *sid2-1* as well as *eds1-1*, and as these proteins are involved in regulating SA levels after pathogen infection, it is possible that a critical threshold level of SA is required for the activation of resistance. To assess this possibility, the level of free SA and SA glucoside (SAG) was monitored in mock- and TCV-inoculated Di-17 and Col-0 plants, as well as in *HRT*-containing F_2 progeny homozygous for the *eds1*, *eds5*, *pad4*, or *sid2* mutations. As a control, SA and SAG levels were also assessed in the Di-17 and Di-17 NahG transgenic plants. Following TCV infection, free SA levels in Di-17 plants increased approximately threefold by 24 h post-inoculation (hpi) and approximately 10-fold by 72 hpi (Figure 2a), while SAG levels increased approximately 40-fold by 72 hpi (Figure 2b). By contrast, SA and SAG levels in susceptible Col-0 plants were two-fold and approximately 40-fold lower, respectively, at 72 hpi than those detected in comparable Di-17 plants. Similarly reduced SA and SAG levels were observed in the TCV-inoculated leaves of *HRT pad4* and *HRT eds5* plants, and even lower levels were detected in *HRT sid2* and Di-17 NahG plants. Surprisingly, SA levels in TCV-inoculated *HRT eds1* plants were only marginally reduced when compared with Di-17 plants, although their SAG levels were as low as those detected in Di-17 NahG plants.

Application of exogenous SA enhances resistance to TCV in an HRT*-specific manner*

To investigate whether SA is a limiting factor for activating TCV resistance in *HRT*-containing plants, Di-17 and Col-0 plants were treated with SA or BTH, and HR development and systemic viral spread were monitored. On Di-17 plants, exogenously applied SA or BTH caused a drastic reduction in the size of the HR lesions, which were only visible as micro-lesions (Figure 3a). A similar decrease in lesion size was also observed in SA/BTH-treated *HRT eds1*, *HRT eds5*, and *HRT sid2* F_2 plants (Figure 3a). By contrast, the phenotype of TCV-inoculated leaves on Col-0 plants was unaffected by SA or BTH treatment (data not shown). In both Col-0 and Di-17 leaves, SA/BTH treatment induced high levels of *PR-1* transcript that were similar to the levels seen in TCV-inoculated Di-17 plants (Figure 3b). BTH treatment also induced *PR-1* gene expression in Di-17 NahG transgenic plants, although it did not restore HR formation (Figure 3b).

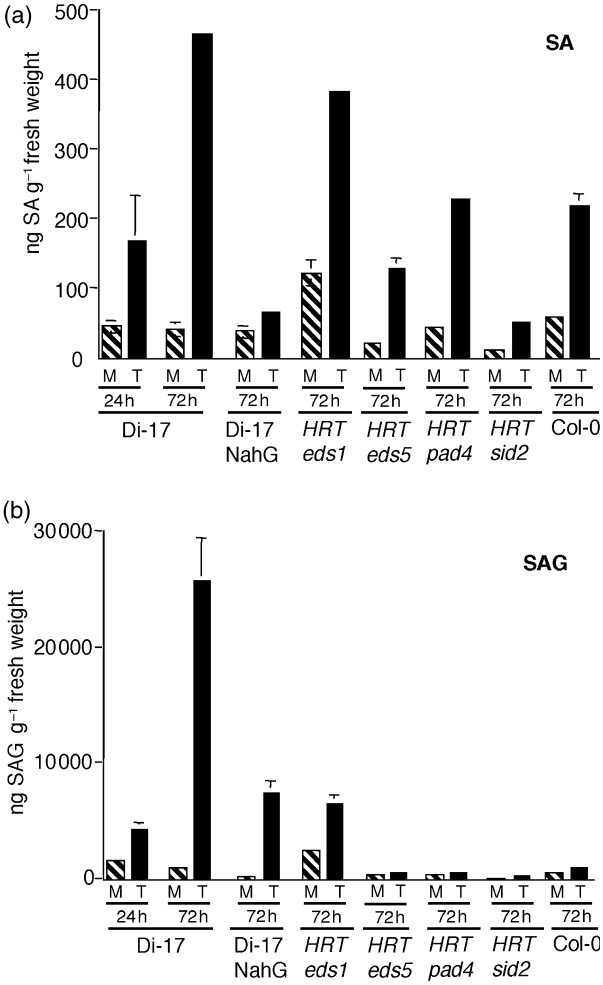

Figure 2. Levels of SA and SAG. Endogenous SA (a) and SAG (b) levels in mock (M) and TCV (T)-inoculated leaves of Di-17, Col-0, Di-17 NahG, *HRT eds1*, *HRT eds5*, *HRT pad4*, and *HRT sid2* plants. Samples were harvested 24 or 72 h post-inoculation.

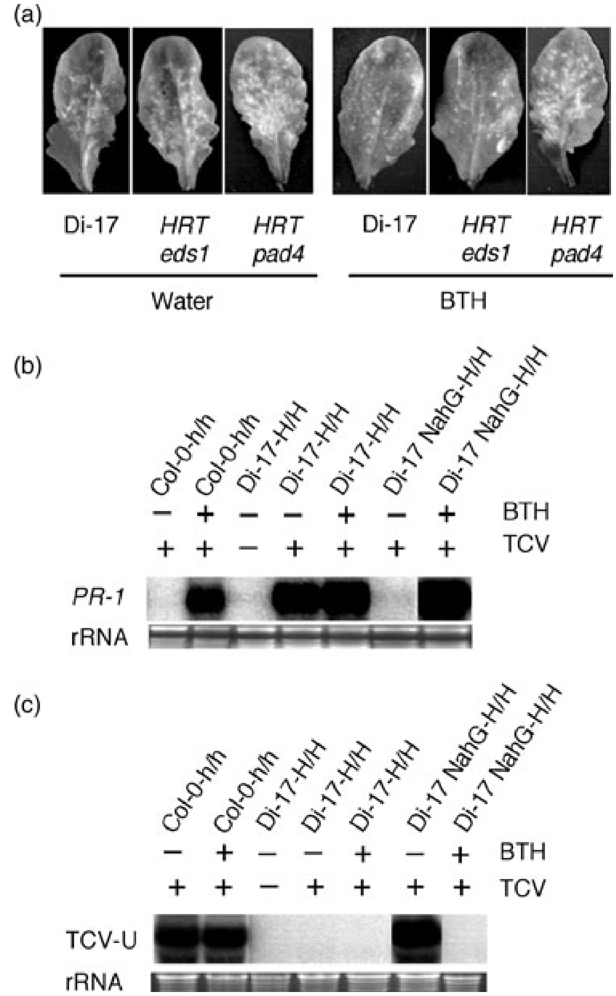

Figure 3. Effect of exogenous application of benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) on the hypersensitive response (HR) and resistance to turnip crinkle virus (TCV).
(a) Cell death in TCV-inoculated Di-17, *HRT eds1* and *HRT pad4* leaves at 4 DPI. Leaves were treated with water or BTH 2 days prior to TCV inoculation. The white specks seen on some leaves are dried inoculation buffer (b) *PR-1* gene expression in water (−)- or BTH (+)-treated and Mock (−)- or TCV (+)-inoculated Col-0, Di-17, and Di-17 NahG plants. Ethidium bromide staining of rRNA was used as a loading control.
(c) Resistance and systemic spread of TCV in various plants shown in (b). RNA was extracted from the uninoculated tissues at 20 DPI and analyzed for the presence of viral transcripts. Ethidium bromide staining of rRNA was used as a loading control.

Turnip crinkle virus resistance in SA/BTH-treated plants was then evaluated by monitoring symptom development and the presence of viral transcripts in uninoculated bolt tissue (Figure 3c). Following SA/BTH treatment, resistance in the Di-17 plants improved from approximately 87 to 97% (Figure 4b). Resistance in the Di-17 NahG plants was also enhanced by BTH treatment; 80% of these plants displayed no disease symptoms and did not accumulate viral transcripts in the uninoculated leaves, whereas the remaining 20% developed milder disease symptoms than water-treated Di-17 NahG plants (Figure 4). SA/BTH treatment also enhanced resistance in the *HRT eds1*, *HRT eds5* and *HRT sid2*, F_2 progeny, and symptom severity was substantially reduced on the susceptible individuals (Figure 4c). However, while *HRT eds1*, *HRT eds5* and *HRT sid2* plants displayed approximately 50–60% resistance following SA/BTH treatment, only approximately 6% of the SA/BTH-treated *HRT pad4* plants were resistant. In comparison, SA/BTH-treated Col-0 plants remained as susceptible as water-treated Col-0 plants. Regardless of the treatment, Col-0 plants displayed similar timing and severity of disease symptoms and accumulated comparable levels of viral transcript in their uninoculated leaves (Figures 3c and 4c).

The observation that SA/BTH treatment enhanced resistance in plants containing the *HRT* gene, but had no effect on Col-0 plants, which lack it, suggested that SA/BTH induces resistance in an *R* gene-dependent manner. To further investigate this possibility, we tested its ability to increase

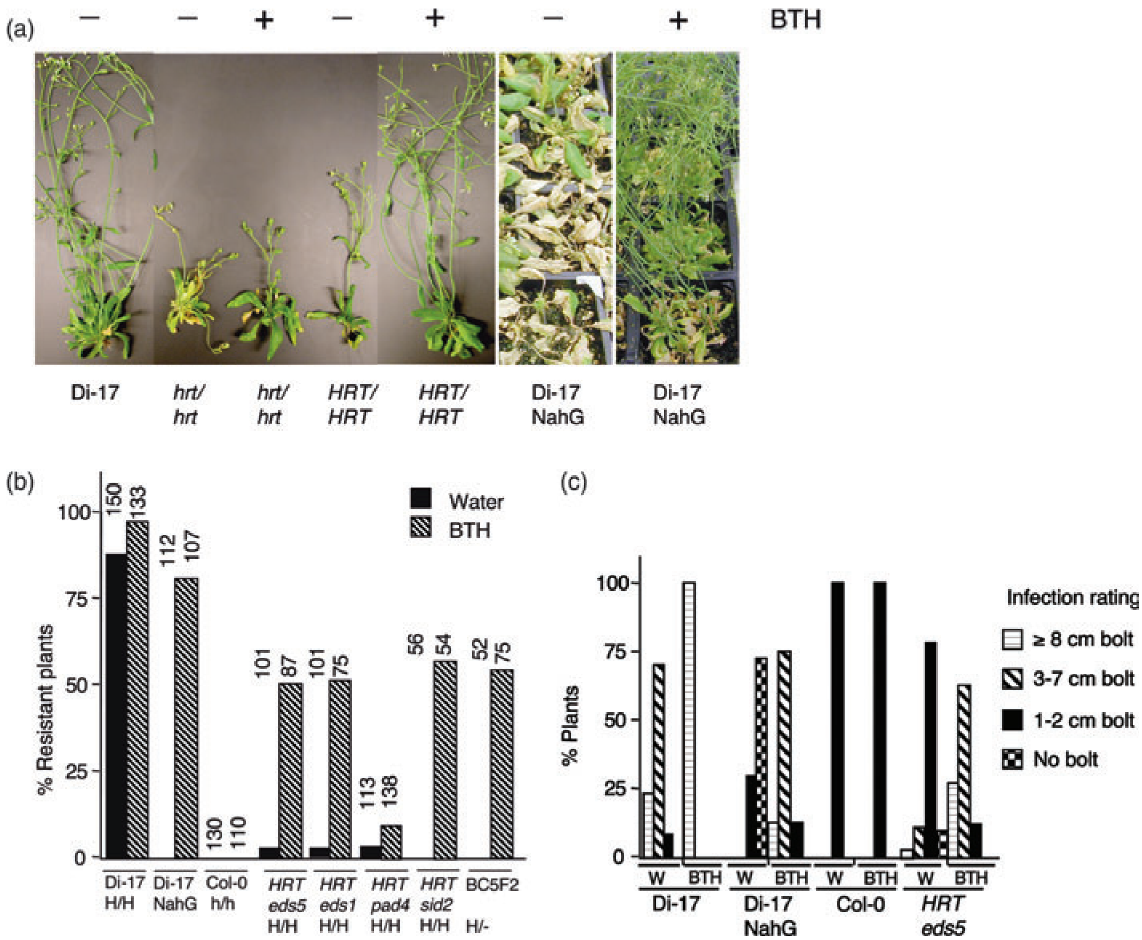

Figure 4. Morphological phenotypes, enhanced resistance and infection rating of water- and benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH)-treated plants inoculated with turnip crinkle virus (TCV).
(a) Morphological phenotypes of TCV-inoculated plants at 21 or 30 DPI.
(b) BTH-induced increase in percentage of the resistant plants. The plants were treated with water or BTH for 2 days prior to TCV inoculations. The number of plants analyzed for each genotype is indicated above each bar.
(c) Infection rating of susceptible plants obtained among certain representative genotypes shown in (b). The severity of disease symptoms was rated according to the key provided on the right.

resistance in the $BC5F_2$ progeny of a cross between Di-17 and Col-0 (see Experimental procedures). As expected, $BC5F_2$ progeny homozygous for *hrt* were uniformly susceptible, exhibiting severe crinkling and stunting, despite SA/BTH treatment. By contrast, resistance in $BC5F_2$ progeny containing at least one copy of *HRT* increased from <1 to 55% following SA/BTH treatment (Table 2, Figure 4b). Taken together, these results confirm that *HRT* is required for SA/BTH treatment to enhance TCV resistance; they also suggest that exogenously supplied SA/BTH can overcome the negative effects of *RRT*.

High endogenous SA levels overcome the suppressive effects of RRT

To further investigate the relationship between *RRT*, TCV resistance and SA levels, we crossed Di-17 with both the *ssi2* mutant [Nössen (Nö) background], which accumulates high levels of endogenous SA (Kachroo *et al.*, 2001, 2003a,b, 2004; Shah *et al.*, 2001), and also wild-type (wt) Nö plants. Both *ssi2* and Nö plants are susceptible to TCV and accumulate high levels of viral transcripts in systemic tissue. Following TCV inoculation, all of the *HRT*-containing F_2 progeny from the Di-17 × Nö cross developed lesions and approximately 25% of these plants were also TCV-resistant. In comparison, TCV-induced cell death was not readily detected on the *HRT ssi2* F_2 progeny from the Di-17 × *ssi2* cross. However, this may be due to the fact that these plants spontaneously develop HR-like lesions or because these plants contain high levels of SA, which reduces the size of the HR lesions (Figure 3a). All of the *HRT ssi2* F_2 progeny were resistant to TCV, as evidenced by normal bolt formation and the lack of systemic viral spread (Table 2, Figure 5). As only 25% of these plants are

Table 2 Segregation of resistance in Di-17 × Nö, Di-17 × *ssi2*, *HRT ssi2*, $BC5F_2$ and *HRT* transgenics E2-8 and E9-4 plants

Cross	Total number of plants analyzed	Genotype[a]	Number of plants obtained	R[b]	S[c]	Hypothesis[d]
Di-17 × Nö	115	*HRT/-*	90	20	70	Two gene segregation, *rrt* is required for resistance
Di-17 × *ssi2*	150	*HRT/-ssi2 ssi2*	30	30	0	One gene segregation, resistance is independent of *rrt*
HRT[e] *ssi2*	40	HRT *ssi2*	40	40	0	One gene segregation, resistance is independent of *rrt*
$BC5F_2$	140	HRT/-	99	0	99	*rrt* is required for resistance
E2-8	56	*HRT/-*	56	0	56	*rrt* is required for resistance
E9-4	47	*HRT/-*	47	46	1	Resistance is independent of *rrt*

[a]The genotype at *HRT* and various mutant loci was determined by CAPS analysis.
[b]Resistant.
[c]Susceptible.
[d]Based on segregation of resistance in *HRT* plants.
[e]F_3 line homozygous for *HRT* and *ssi2*.

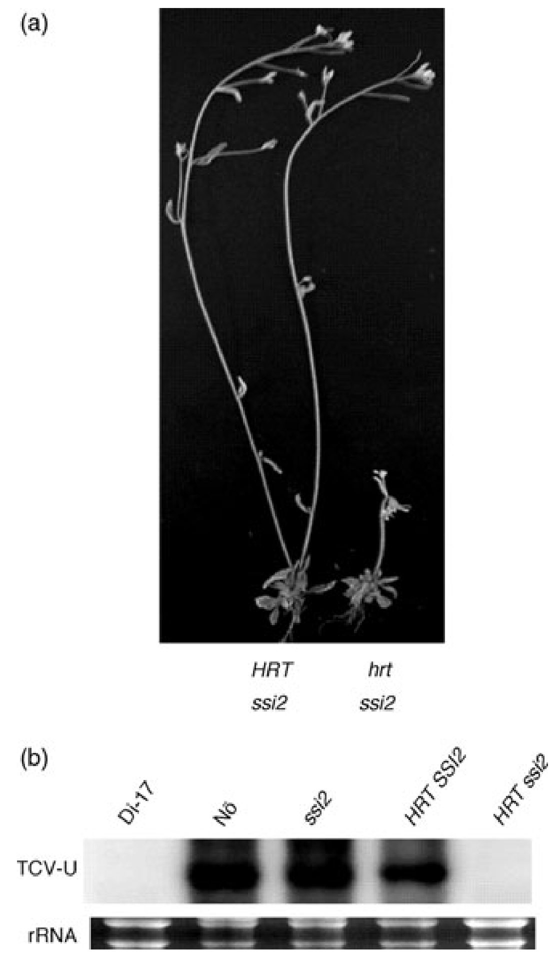

Figure 5. Effect of high endogenous salicylic acid on resistance to turnip crinkle virus (TCV).
(a) Morphological phenotypes of *HRTssi2*, and *hrt ssi2* plants at 14 DPI. The *hrt ssi2* plants show typical crinkling and stunted bolt development phenotype seen in susceptible plants.
(b) Systemic spread of TCV to uninoculated tissue in Di-17, Nö, *ssi2*, *HRT SSI2* and *HRT ssi2* plants. RNA was extracted from the uninoculated tissues at 18 DPI and analyzed for the presence of viral transcripts. Ethidium bromide staining of rRNA was used as a loading control.

predicted to be resistant, this result suggests that high levels of *ssi2*-generated SA overcame the suppressive effects of *RRT*. Moreover, as *hrt ssi2* F_2 progeny did not develop an HR and were completely susceptible to TCV infection (Figure 5), the elevated endogenous SA levels conferred by *ssi2* appear to activate TCV resistance in an *HRT*-dependent manner.

SA upregulates HRT *expression levels in a* PAD4*-dependent manner*

Salicylic acid induces expression of several *R* genes, including *SSI4*, *RPP1*, and *RPS4* (Shirano *et al.*, 2002). This finding, combined with the demonstration that *HRT* copy number positively influences TCV resistance (Cooley *et al.*, 2000; Kachroo *et al.*, 2000), raised the possibility that SA/BTH treatment enhances TCV resistance by upregulating *HRT* expression. Consistent with this hypothesis, SA and/or BTH application increased both *HRT* transcript accumulation and resistance in Di-17 plants and *HRT sid2*, *HRT eds1*, and *HRT eds5* F_2 plants (Figures 4b and 6a). By contrast, *HRT* expression was not induced in SA-treated *HRT pad4* plants, which displayed only a small increase in resistance following BTH treatment. One explanation for this result is that the *pad4* mutation, unlike *sid2*, *eds1*, and *eds5*, might affect the TCV resistance signaling pathway downstream of SA. However, SA-induced *PR-1* expression was comparable in all of the mutants, as well as in Di-17 and Col-0 plants (Figure 6b), arguing that this possibility is unlikely. Alternatively, *PAD4* may be required for SA-mediated upregulation of *HRT* expression.

Analysis of SA-treated Col-0 plants and *ssi2* (Nö background) and *cpr5* (Col-0 background) mutants, which constitutively accumulate high levels of SA, revealed that exogenously or endogenously supplied SA does not induce elevated expression of the *hrt* allele (Figure 6a). Only basal level *hrt* expression was detected in these plants, although elevated *HRT* expression was detected in

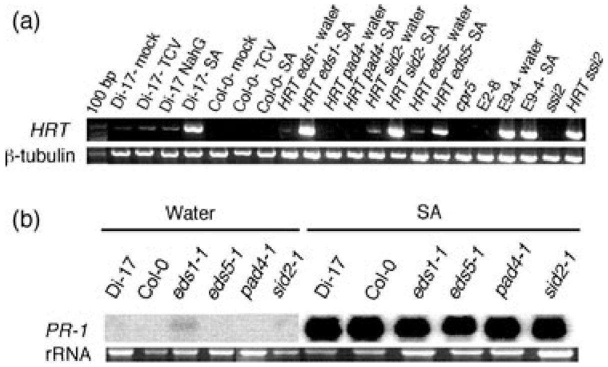

Figure 6. Salicylic acid (SA)- or turnip crinkle virus (TCV)-induced expression of *HRT* and *PR-1*.
(a) RT-PCR analysis of plants treated with SA or water. Plants were sprayed with SA or water or inoculated with TCV, and leaves were harvested 48 h post-water or SA treatments and 72 h post-TCV inoculation. RT-PCR was performed using total RNA and *HRT* gene-specific primers (Cooley *et al.*, 2000) and the products were visualized on an ethidium bromide-stained agarose gel. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template.
(b) SA-induced expression of *PR-1* in Di-17, Col-0, *eds1*, *eds5*, *pad4*, and *sid2* backgrounds. The plants were sprayed with water or SA at 48 h prior to harvesting the samples for RNA extractions. Ethidium bromide staining of rRNA was used as a loading control.

HRT ssi2 plants. Taken together, these data suggest that the *HRT* allele is SA-inducible whereas the recessive *hrt* allele is not.

The correlation between elevated *HRT* expression and resistance was further investigated using Col-0-*HRT* transgenic lines. Similar to previous results (Cooley *et al.*, 2000), the majority of these Col-0-*HRT* transgenic lines, including E2-8, developed a normal HR after TCV infection but then developed disease symptoms and died 14–21 days post-infection. However, a small percentage of transgenic lines, including E9-4, produced a micro-HR after TCV inoculation and did not allow systemic viral spread (Table 2). RT-PCR analysis revealed that E2-8 expressed *HRT* at low levels similar to those detected in water- or SA-treated Col-0 plants (Figure 6a). By contrast, water-treated E9-4 plants accumulated high levels of *HRT* transcripts that were comparable to those observed in SA-treated Di-17 plants. Taken together, these results indicate that high-level *HRT* expression correlates with TCV resistance. Furthermore, these results suggest that the mechanism through which SA enhances resistance even in the presence of *RRT* involves activating high-level *HRT* expression.

An inverse correlation was also observed between *HRT* levels and the HR to TCV; *HRT ssi2*, E9-4 or SA-treated *HRT* plants show a drastic reduction in the size of HR lesions upon TCV inoculation. This was further assessed by analyzing cell death in SA-treated *HRT pad4* plants, which are unable to accumulate high levels of *HRT*. Interestingly, both water- and SA-treated *HRT pad4* plants showed similar HR

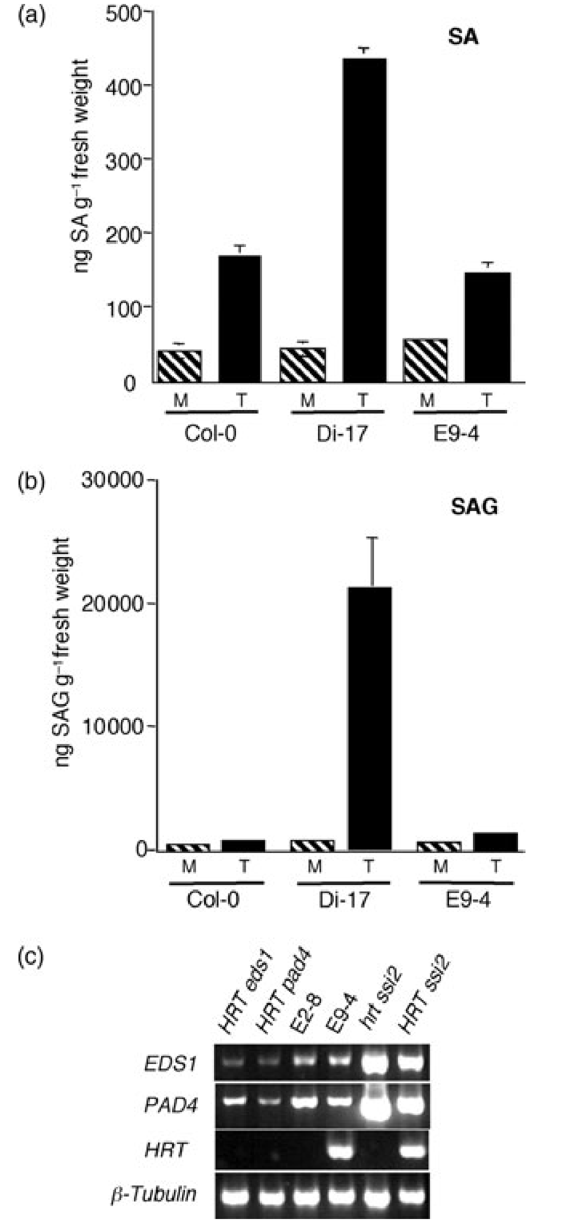

Figure 7. Levels of salicylic acid (SA) and salicylic acid glucoside (SAG) and SA-induced expression of *EDS1* and *PAD4*. Endogenous SA (a) and SAG (b) levels in mock (M)- and turnip crinkle virus (TCV) (T)-inoculated leaves of Col-0, Di-17, and E9-4 plants. Samples were harvested 72 h post-inoculation.
(c) RT-PCR analysis of *HRT eds1*, *HRT pad4*, E9-4, E2-8, *hrt ssi2*, and *HRT ssi2* plants. RT-PCR was performed using total RNA and *EDS1* and *PAD4* gene-specific primers and the products were visualized on an ethidium bromide-stained agarose gel. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template.

on TCV-inoculated leaves (Figure 3a). These results provide additional evidence that overexpression of *HRT* is responsible for a reduction in the size of HR lesions and that a mutation in *pad4* abolishes SA-mediated increase in *HRT* transcript.

Increased expression of HRT *does not alter SA levels*

The positive correlation between SA content and *HRT* transcript levels suggested that increased *HRT* expression might lead to enhanced resistance by increasing the SA levels. To assess this possibility, SA levels were monitored in Col-0-*HRT* transgenic lines exhibiting high (E9-4) and basal (E2-8) levels of the *HRT* transcript (Figure 7a). Following mock inoculation, both lines contained similar levels of SA and SAG as the Col-0 parental line, as well as Di-17 plants (Figure 7). Following TCV inoculation, SA and SAG levels in E9-4 plants increased to similar levels as those detected in Col-0 plants. These results indicate that elevated expression of *HRT* does not lead to constitutive SA accumulation. Rather, it appears that SA synthesis is induced by the combined presence of *HRT* and TCV. Furthermore, overexpression of *HRT* in E9-4 plants also does not elevate the expression of *EDS1* or *PAD4* genes (Figure 7c). However, *HRT ssi2* plants showed increased expression of both *EDS1* and *PAD4* genes, which is likely the result of feedback induction of these genes by high levels of SA in *ssi2* and *HRT ssi2* plants (Figure 7c).

Discussion

In this study, we conducted an extensive analysis of the signaling pathway through which *Arabidopsis* resists infection by TCV. Consistent with previous results, TCV resistance required the *R* gene, *HRT*, and a recessive allele of *rrt*. Epistasis analyses further revealed that *HRT*-mediated *PR-1* gene expression and cell death are independent of *EDS1*, *EDS5*, *PAD4*, *NDR1*, *RAR1*, *SGT1*, or *SID2*, whereas *HRT/rrt*-mediated TCV resistance requires *EDS1*, *EDS5*, *PAD4*, and *SID2*, but is unaffected by mutations in *rar1*, *sgt1b*, or *ndr1*. *EDS1*, *EDS5*, *PAD4*, and *SID2* all play a role in pathogen-induced SA accumulation (Falk *et al.*, 1999; Jirage *et al.*, 1999; Nawrath and Metraux, 1999; Wildermuth *et al.*, 2001). This shared characteristic combined with the demonstration that TCV resistance is suppressed in Di-17 NahG plants (Kachroo *et al.*, 2000), suggests that the *eds1*, *eds5*, *pad4*, and *sid2* mutations compromised resistance by lowering SA levels. In line with this, *HRT*-containing F_2 plants homozygous for these mutations accumulated reduced levels of SA following TCV inoculation. Further evidence that SA plays a role in *HRT* signaling comes from the demonstration that treating *Arabidopsis* plants with SA or its functional analog BTH enhanced TCV resistance. Strikingly, while SA or BTH treatment conferred high levels of resistance to Di-17 and *HRT*-containing *eds1*, *eds5*, and *sid2* plants, it had no effect on the susceptibility of Col-0 plants or *hrt* homozygous F_3 progeny from a Di-17 $\times$ Col-0 cross. SA therefore appears to enhance resistance by acting in conjunction with *HRT* or an *HRT*-derived signal.

The finding that *HRT* is dependent on EDS1 and independent of NDR1 was highly unexpected, as *R* genes with a CC–NBS–LRR structure, such as *HRT*, usually require *NDR1* to signal resistance responses, while *R* genes with a TIR–NBS–LRR structure utilize *EDS1* (Aarts *et al.*, 1998; Dangl and Jones, 2001). A few *R* genes, such as *RPP7* and *RPP8*, appear to be independent of both *NDR1* and *EDS1* (Aarts *et al.*, 1998; McDowell *et al.*, 2000). EDS1 may regulate resistance to TCV either via a downstream signaling event, or by maintaining SA levels or by a combination of the two. As resistance in *HRT eds1* plants can be enhanced by exogenous application of SA, it is likely that EDS1 functions as a general feedback loop that participates in resistance signaling by way of maintaining SA levels. However, as SA is known to act downstream of EDS1, it is equally likely that exogenous application of SA bypasses a requirement for EDS1. Further studies are therefore required to decipher the role of EDS1 in HRT-mediated resistance to TCV. As resistance to TCV is dependent on the ability of the plant to prevent spread of the virus into systemic tissue, it will be equally important to establish whether or not EDS1 plays a similar role in systemic versus inoculated tissues.

In comparison with TCV resistance, *HRT*-mediated cell death and *PR-1* expression were unaffected by mutations in *eds1*, *eds5*, *pad4*, or *sid2*. As *HRT sid2* plants accumulated very low levels of SA following TCV infection, cell death and *PR-1* expression appear to be activated via an SA-independent pathway(s). This result conflicts with our previous demonstration that cell death and *PR-1* expression are suppressed in Di-17 NahG plants (Kachroo *et al.*, 2000). Other studies have also detected differences between the resistance phenotype of NahG plants and other SA-deficient plants such as *eds1*, *eds5*, *pad4*, and/or *sid2* mutants (Heck *et al.*, 2003; van Wees and Glazebrook, 2003). One explanation for this discrepancy is that catechol, which is produced by SA degredation in NahG plants, affects resistance signaling. In line with this, catechol compromises resistance to *Pseudomonas syringae* pv. *maculicola* (van Wees and Glazebrook, 2003). In addition, TCV-inoculated Di-17 plants treated with catechol develop fewer lesions than comparable water-treated plants (data not shown). Thus, we suspect that catechol accumulation, rather than reduced SA levels, is responsible for the suppression of TCV-induced cell death and *PR-1* expression in Di-17 NahG plants.

Earlier genetic and transgenic analyses revealed that moderate- to low-level *HRT* expression in the Col-0 and Nö ecotypic backgrounds is insufficient to confer resistance to TCV, due to the suppressive effects of a dominant gene, *RRT* (Cooley *et al.*, 2000; Kachroo *et al.*, 2000). However, SA treatment enhanced resistance in an *HRT*-dependent manner in the F_3 progeny of a cross between Di-17 and Col-0 and in the $BC5F_2$ population. High endogenous SA levels produced in the *ssi2* mutant also conferred enhanced resistance in the *RRT* background, provided an *HRT* allele was present. Analysis of *HRT ssi2* plants and SA-treated Di-17, *HRT eds1*, *HRT eds5*, and *HRT sid2* plants revealed a correlation between TCV resistance and heightened *HRT* expression. Thus, we hypothesized that exogenously supplied SA or high levels of endogenous SA induced resistance in the *RRT* background by stimulating *HRT* expression. Consistent with this conclusion, overexpression of *R* genes confers enhanced pathogen resistance (Oldroyd and Staskawicz, 1998; Stokes *et al.*, 2002; Tang *et al.*, 1999), and SA treatment induces the expression of several *R* genes (Maleck *et al.*, 2000; Shirano *et al.*, 2002). Moreover, Col-0 transgenic lines expressing high levels of *HRT* are TCV-resistant whereas those expressing moderate to low levels of *HRT* are susceptible. While these results strongly suggest that the mechanism through which SA overcomes the suppressive effect of *RRT* involves increasing *HRT* expression, it should be noted that SA induced only TIR–NBS–LRR type *R* gene expression (Shirano *et al.*, 2002). As SA did not activate expression of the CC–NBS–LRR *R* genes *RPM1* or *RPS2*, its ability to upregulate *HRT* expression is novel.

In comparison with SA-treated *HRT eds1*, *HRT eds5*, and *HRT sid2* F_2 plants, SA-treated *HRT pad4* plants displayed low levels of TCV resistance and failed to express enhanced levels of *HRT*. SA-induced *PR* gene expression was unaffected in the *pad4* mutant, suggesting that *HRT pad4* plants are not SA-insensitive. Instead, loss of *PAD4* appears to suppress SA-induced *HRT* upregulation, which would explain why SA treatment does not restore TCV resistance effectively in these plants. Analysis of E9-4 Col-0 transgenics, which express high levels of *HRT*, further revealed that while SA upregulates *HRT* expression, high-level *HRT* expression does not confer constitutive SA accumulation. SA levels in E9-4 plants only increased after TCV infection, suggesting that *HRT* stimulates this response only in the presence of the TCV coat protein, which is the avirulence determinant for this virus (Oh *et al.*, 1995; Zhao *et al.*, 2000).

The observation that TCV resistance in Col-0 transgenic plants correlates with *HRT* expression levels suggests that RRT suppresses resistance by directly or indirectly blocking HRT function (Figure 8). Perhaps in plants containing high levels of HRT, the RRT repressor is titrated out thereby allowing the excess HRT to work with SA to activate TCV resistance. By contrast, if only low levels of HRT are present, their activity is effectively suppressed by RRT. Alternatively, RRT might function by blocking SA accumulation and/or action. However, RRT does not appear to block SA accumulation, as SA levels in the *HRT*-containing $BC5F_2$ progeny were comparable with those in Di-17 plants (Figure S1). RRT also does not appear to suppress resistance by blocking SA action; similar levels of SA (and presumably RRT) were detected in resistant E9-4 transgenics and susceptible Col-0 parental plants following TCV inoculation. Additionally, the

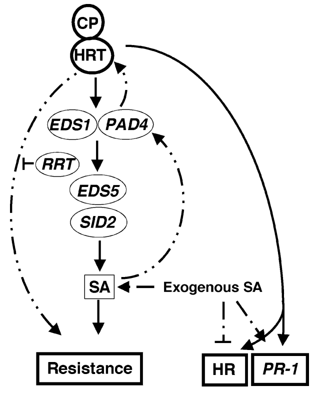

Figure 8. Model for induction of the hypersensitive response (HR) and resistance to turnip crinkle virus (TCV).
TCV-induced cell death is initiated upon direct or indirect interaction between the dominant resistance protein HRT and TCV's avirulence factor, the coat protein (CP). Upon recognition of the pathogen, an *HRT*-mediated response leads to the accumulation of SA, which is dependent on the *EDS1*, *PAD4*, *EDS5*, and *SID2* genes. In contrast, the HR and *PR-1* gene expression are independent of *EDS1*, *PAD4*, *EDS5*, and *SID2* genes. These phenotypes also appear to be SA-independent, as they remain unaffected in mutant backgrounds, which cause moderate or drastic reductions in SA levels. As *RRT* appears to suppress *HRT*-mediated resistance but not the increase in SA induced by TCV infection, it is likely to function downstream or independent of the SA pathway. SA upregulates expression of both *EDS1* and *PAD4* and thus forms a signal-amplification loop with these genes (Falk *et al.*, 1999; Feys *et al.*, 2001; Jirage *et al.*, 1999). Exogenous application of SA also upregulates expression of the *HRT* and this step is mediated via *PAD4* (shown by dash and dotted lines). Exogenous application of SA induces *PR-1* gene expression in Di-17 plants and also leads to suppression of HR.

ability of exogenous SA to induce *HRT* and *PAD4* expression was unaffected in the *HRT*-containing $BC5F_2$ population, which presumably carry the *RRT* allele.

It is interesting to note that while high-level *HRT* expression appears to be required for TCV resistance in the *RRT* background (Cooley *et al.*, 2000), it is not associated with resistance in Di-17 plants. However, we cannot rule out the possibility that TCV induces *HRT* expression to a low level in Di-17 plants or that induction occurs at a time point other than 12, 24, 48, or 72 h post-inoculations. Presumably, the basal levels or a low-level induction of HRT in Di-17 plants (Cooley *et al.*, 2000) are sufficient to activate resistance in the absence of the RRT suppressor, as long as appropriate SA levels are available. One possible explanation for the 2–15% susceptibility observed in Di-17 plants is that these individuals fail to express sufficient levels of HRT and/or to accumulate enough SA to activate resistance. In line with this, SA treatment induces *HRT* expression and confers nearly 100% resistance in Di-17 plants.

The mechanism through which *HRT* works with SA to activate TCV resistance is currently unclear. Further studies are therefore required to determine whether HRT and SA work together to activate TCV resistance through one or more of these mechanisms, or whether they activate viral resistance via a pathway that is yet to be defined.

Experimental procedures

Plant growth conditions and viral infections

Plants were grown in the MTPS 144 Conviron walk-in-chambers at 22°C, 65% relative humidity and 14 h photoperiod. Transcripts synthesized *in vitro* from a cloned cDNA of TCV using T7 RNA polymerase were used for viral infections (Dempsey *et al.*, 1993; Oh *et al.*, 1995). For inoculations, the viral transcript was suspended at a concentration of 0.05 $\mu g\ \mu l^{-1}$ in inoculation buffer, and the inoculation was performed as described earlier (Dempsey *et al.*, 1993). After viral inoculations, the plants were transferred to a Conviron MTR30 reach-in chamber maintained at 22°C, 65% relative humidity and 14 h photoperiod. Cell death was determined visually 3–4 days post-inoculation (DPI). Resistance and susceptibility were scored at 14–21 DPI and confirmed by Northern gel blot analysis. Susceptible plants showed stunted growth, crinkling of leaves, and drooping of the bolt.

Chemical treatment of plants

Three-week-old plants were sprayed or subirrigated with a solution of 500 μM SA or 100 μM BTH. Control plants were treated with water and 2 days after treatment, three leaves per plant were inoculated with TCV RNA.

SA and SAG estimations

Salicylic acid extraction was based on the method of Gaffney *et al.* (1993) with modifications to allow for a high-throughput approach and recovery. Anisic acid was used as an internal standard and SA recovery averaged greater than 80%. Results are the average of three to nine independent extractions. Samples were analyzed on an Agilent 1100 with DAD and FLD detection, using a Novapak C18 column (Waters, Milford, MA, USA).

RNA extraction and gel analysis

Small-scale RNA extraction was performed with TRIzol reagent (Invitrogen, Rockville, MD, USA), according to the manufacturer's instructions. RNA gel blot analysis and synthesis of random primed probes were performed as described earlier (Kachroo *et al.*, 2000).

Transgenic and genetic analyses

The Di-17 NahG plants were created by transforming Di-17 plants with the pCIB200-NahG construct, which contains the salicylate hydroxylase gene from *Pseudomonas putida* under the control of the 35S promoter. The transgenic plants were selected on MS medium supplemented with 50 $\mu g\ ml^{-1}$ of kanamycin. The Col-0 transgenic plants containing a genomic copy of *HRT* present within approximately 40 kb fragment (At5g43470; Cooley *et al.*, 2000) were screened for resistance to TCV in the F_3 and F_4 generations. A majority of the transgenic lines produced an HR, expressed *PR-1* in high amounts, and allowed systemic spread of the virus. A few of the transgenic lines did not produce an HR and showed absolute resistance to TCV. Two lines, E9-4 and E2-8, scored as resistant and susceptible to TCV, respectively, were characterized further.

The BC5F_2 population was derived upon backcrossing an *HRT* homozygous F_2 plant (derived from a cross between Di-17 and Col-0) with the susceptible Col-0 parent, which was used as a recurring parent for five more backcrosses. The backcrossed F_2 progeny were scored for TCV resistance after third backcross and found to be uniformly susceptible.

Crosses were performed by pollinating flowers of Di-17 plants with pollen from Col-0, Nö, Ws, *eds1*, *pad4*, *eds5*, *sid2*, *rar1*, *ndr1*, *sgt1b*, and *ssi2* plants. The genotypes of the F_2 plants at the *HRT*, *NDR1*, *EDS1*, *RAR1*, *SGT1*, *PAD4*, and *SID2* loci were determined by conducting cleaved amplified polymorphic sequence (CAPS) analysis (Cooley *et al.*, 2000; Kachroo *et al.*, 2000). Because the *eds5-1* mutation does not lead to an alteration of any restriction site, we used the dCAPS technique (Neff *et al.*, 1998) to generate polymorphism between the wild-type and *eds5-1* alleles. A 100-bp region encompassing the mutant base was amplified using primers CAAATCAACATTTGT TTCCTGTGTTT TTG and CATGAAGAAAGGTAT AAGCAGTCTATGGAT, and digested with *Sau*3AI. The digested PCR product amplified from *eds5-1* generated a single band at 100 bp, whereas the wild-type allele yielded two bands at 75 and 25 bp. The plants containing the NahG transgene were identified using PCR analysis as described before (Kachroo *et al.*, 2000). *ssi2* homozygous plants were identified using PCR analysis as described before (Kachroo *et al.*, 2001).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2241/TPJ2241sm.htm.

Figure S1. SA levels in BC5F_2 plants.

Endogenous total SA levels in mock (M) and TCV (T)-inoculated leaves of Col-0, Di-17, and BC5F_2 plants. Samples were harvested 72 h post-inoculation.

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